

University of Dundee

## Potential Role of Brain-Derived Neurotrophic Factor and Dopamine Receptor D2 Gene Variants as Modifiers for the Susceptibility and Clinical Course of Wilson's Disease

Roy, Shubhrajit; Pal, Prosenjit; Ghosh, Sampurna; Bhattacharya, Sreyashi; Das, Shyamal Kumar; Gangopadhyay, Prasanta Kumar

*Published in:*  
NeuroMolecular Medicine

*DOI:*  
[10.1007/s12017-018-8501-2](https://doi.org/10.1007/s12017-018-8501-2)

*Publication date:*  
2018

*Document Version*  
Peer reviewed version

[Link to publication in Discovery Research Portal](#)

*Citation for published version (APA):*

Roy, S., Pal, P., Ghosh, S., Bhattacharya, S., Das, S. K., Gangopadhyay, P. K., Bavdekar, A., Ray, K., Sengupta, M., & Ray, J. (2018). Potential Role of Brain-Derived Neurotrophic Factor and Dopamine Receptor D2 Gene Variants as Modifiers for the Susceptibility and Clinical Course of Wilson's Disease. *NeuroMolecular Medicine*, 20(3), 401-408. <https://doi.org/10.1007/s12017-018-8501-2>

### General rights

Copyright and moral rights for the publications made accessible in Discovery Research Portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from Discovery Research Portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain.
- You may freely distribute the URL identifying the publication in the public portal.

### Take down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Potential role of brain-derived neurotrophic factor and dopamine receptor D2 gene variants as modifiers for the susceptibility and clinical course of Wilson's disease

Shubhrajit Roy<sup>1</sup>, Prosenjit Pal<sup>1</sup>, Sampurna Ghosh<sup>2</sup>, Sreyashi Bhattacharya<sup>2</sup>, Shyamal Kumar Das<sup>3</sup>, Prasanta Kumar Gangopadhyay<sup>4</sup>, Ashish Bavdekar<sup>5</sup>, Kunal Ray<sup>6</sup>, Mainak Sengupta<sup>2</sup>, Jharna Ray<sup>1</sup>

<sup>1</sup>S. N. Pradhan Centre for Neurosciences, University of Calcutta, Kolkata; <sup>2</sup>Department of Genetics, University of Calcutta, Kolkata; <sup>3</sup>Bangur Institute of Neurosciences, Kolkata; <sup>4</sup>Calcutta National Medical College, Kolkata; <sup>5</sup>KEM Hospital, Pune; <sup>6</sup>Academy of Scientific and Innovative Research (AcSIR), New Delhi, India

Corresponding Authors

Prof. Jharna Ray  
S. N. Pradhan Centre for Neurosciences  
University of Calcutta  
35, Ballygunge Circular Road  
Kolkata – 700019  
West Bengal  
India  
E-mail ID: [jharnaray@gmail.com](mailto:jharnaray@gmail.com)

Dr. Mainak Sengupta\*  
Department of Genetics  
University of Calcutta  
35, Ballygunge Circular Road  
Kolkata – 700019  
West Bengal  
India  
E-mail ID: [sengupta.mainak@gmail.com](mailto:sengupta.mainak@gmail.com)

\*All correspondence with the Journal will be done by Dr. Mainak Sengupta

## Abstract:

**Background:** Wilson's disease (WD), an inborn error of copper metabolism caused by mutations in the ATPase copper transporting beta (*ATP7B*) gene, manifests variable age of onset and different degrees of hepatic and neurological disturbances. This complex phenotypical outcome of a classical monogenic disease can possibly be explained by modifier loci regulating the clinical course of the disease. The brain-derived neurotrophic factor (BDNF), critical for the survival, morphogenesis and plasticity of the neurons, and the dopamine receptor D2 (DRD2), one of the most abundant dopamine receptors in the brain, have been highlighted in the pathophysiology of various neuropsychiatric diseases. This study aims to identify the potential association between *BDNF* and *DRD2* gene polymorphisms and WD and its clinical characteristics.

**Methods:** A total of 164 WD patients and 270 controls from India were included in this study. Two *BDNF* polymorphisms [p.Val66Met (c.G196A) and c.C270T] and the *DRD2* Taq1A (A2/A1 or C/T) polymorphism were examined for their association with WD and some of its clinical attributes, using polymerase chain reaction, restriction fragment length digestion and bidirectional sequencing.

**Results:** The C allele and CC genotype of *BDNF* C270T were significantly overrepresented among controls compared to WD patients. In addition, a significantly higher proportion of the allele coding for Val and the corresponding homozygous genotype of *BDNF* Val66Met polymorphism was found among WD patients with age of onset later than 10 years. Furthermore, the A1A1 genotype of *DRD2* Taq1A polymorphism was significantly more common among WD patients with rigidity.

**Conclusion:** Our data suggest that both *BDNF* and *DRD2* may act as potential modifiers of WD phenotype in the Indian context.

**Key words:** *BDNF*, *DRD2*, Wilson's disease, WD

## Introduction:

Wilson's disease (WD) is an autosomal recessive disorder of copper metabolism caused by mutations in the ATPase copper transporting beta (*ATP7B*) gene, which encodes a copper-transporting P-type ATPase primarily expressed in liver and brain. A broad spectrum of mutations in *ATP7B* leads to toxic deposition of copper in liver and brain giving rise to a wide-range of hepatic and neurological features, such as, low plasma ceruloplasmin level, high urinary copper, dystonia, Parkinsonism, gait disturbances, rigidity along with Kayser Fleischer ring (K - F ring) (Das and Ray, 2006). A large number of mutations have been reported in *ATP7B* (Todorov et al, 2016). Interestingly significant phenotypic variation has been noticed between monozygotic twins with the same *ATP7B* mutation highlighting the potential involvement of modifier loci (Kegley et al, 2010). Potential modifier genes, such as, apolipoprotein E (*APOE*), human prion related protein (*PRNP*), methylenetetrahydrofolate reductase (*MTHFR*) and divalent metal transporter 1 (*DMT1*) have been proposed to be associated with the clinical course of the disease (Kieffer and Medici V, 2017).

Brain derived neurotrophic factor (BDNF), is essential for differentiation and survival of the neurons, modulating synaptic plasticity and synaptic transmission (Mizui et al, 2014). It regulates long term potentiation and long term depression functions in the hippocampal and cortical neurons, thus affecting learning and memory (Mizui et al, 2015). Metal ions may influence the BDNF activity and it is well known that the alteration of Cu<sup>2+</sup> homeostasis is a prominent factor in the development of neuropathologies (Travaglia et al, 2012). BDNF is initially synthesized as a precursor protein (i.e. prepro-BDNF) in the endoplasmic reticulum. Following cleavage of the signal peptide, pro-BDNF is converted to mature BDNF and BDNF pro-peptide. BDNF as dimer binds to two receptors of distinct classes: the type B tyrosine kinase receptor of the tropomyosin-related kinase family (TrkB) and the p75 receptor, which is a member of the tumor necrosis factor (TNF) receptor family (Mizui et al, 2014). Previous reports suggest that *BDNF* polymorphisms and reduced BDNF expression in human brains are closely related to the pathogenesis of several neurodegenerative and neuropsychiatric disorders (Autry and Monteggia, 2012).

A common single-nucleotide polymorphism (SNP) in *BDNF*, rs6265 (c.G196A), which results in a change of valine to methionine amino acid at codon 66, presents in the pro-domain region of the BDNF pro-peptide serving as a bioactive molecule for neurons. This SNP has been associated with abnormalities in memory, learning and neuropsychiatric functions (Mizui et al, 2015; Mizui et al, 2017). A functional promoter polymorphism, rs56164415 (c.C270T) of the *BDNF* gene has been extensively studied in schizophrenia and Alzheimer's disease (Watanabe et al, 2013). Bioinformatics analysis revealed that C>T substitution causes loss of transcription factor binding, which may alter the transcriptional efficacy (Catharius et al, 2005).

Dopamine receptor D2 (DRD2) is encoded by the *DRD2* gene, which is located on chromosome 11q22.2 in humans (Klein et al, 1999). Reduced striatal dopamine levels have been observed in WD patients (Nyberg et al, 1982). Animal studies have indicated

decreased DRD2 expression during copper overloading (De Vries et al, 1986). Single photon emission computerized tomography (SPECT) and positron emission tomography (PET) studies have shown postsynaptic loss of dopamine D2 receptors in striatum of WD patients (Oder et al, 1996, Westermarck et al, 1996). Interestingly, WD patients also exhibit reduced bindings of dopamine ligands to dopamine receptors on lymphocytes probably due to damaged dopamine receptors resulting from copper toxicity (Członkowska et al, 1987). *DRD2* has been found to be associated with Parkinson's disease, dystonia, addiction and schizophrenia (Noble et al, 2003).

The Taq1A polymorphism (dbSNP rs1800497; A2/A1 or C/T), located ~9.5 kb downstream from *DRD2* in exon 8 of ankyrin repeat and kinase domain containing 1 (*ANKK1*) gene, has been investigated extensively (Blum et al, 2017) in neurological diseases. The A1 (T) allele of this polymorphism has been associated with reduced density of striatal dopamine D2 receptors and has been associated with increased risk of Alzheimer's disease among African Americans (Blum et al, 2017). It is worth mentioning that the Hap-Map project revealed that the studied Taq1A polymorphism, although present in the exon 8 of *ANKK1* gene, downstream of *DRD2* locus, is in linkage disequilibrium (LD) with other *DRD2* variants but not with any of the *ANKK1* polymorphisms, and has therefore been considered to have regulatory implications for *DRD2* expression (Munafo et al, 2007).

So far, about 150 *ATP7B* mutations have been identified in Indian WD patients (<http://www.igdd.iicb.res.in/IGDD/home.aspx>). Interestingly, a *Copper metabolism MURR1 domain containing protein 1 (COMMD1)* mutation has been associated with abnormally high urinary copper in one patient (Gupta et al, 2010). Again, we have found association of *APOE* and *PRNP* polymorphisms as modifiers of susceptibility and clinical features among Indian WD patients (Roy et al, 2017). However, all the variable clinical features of WD cannot be explained on the basis of these genes. The roles of *BDNF* and *DRD2* polymorphisms have not been tested among Indian WD patients for their potential to modify the clinical course of WD. Hence, the objective of this study was to test the association between genetic variations in *BDNF* and *DRD2* with the susceptibility and clinical features of WD in the Indian context.

## Materials and Methods:

**Study Subjects:** A total of 164 symptomatic unrelated WD patients comprising of 60 female and 104 male individuals participated in this study. Clinical and biochemical evaluations of the patients were performed at the Bangur Institute of Neurosciences, Kolkata; the National Medical College, Kolkata, West Bengal, India (n = 142); and the King Edward Memorial Hospital, Pune, Maharashtra, India (n = 22) as previously described (Mukherjee et al, 2014). A total of 270 healthy individuals (58 female and 212 male) lacking any personal or family history of neurological diseases, collected from general population from Kolkata, India served as controls. The controls and WD patients were mostly from the Indo-European linguistic group. Informed written consent was provided by all the participants. WD patients' blood collection, DNA isolation and screening of *ATP7B* were done previously as described (Mukherjee et al, 2014). The diagnosis of WD patients was done following Sternleib's criteria (Sternleib, 1990). Decreased serum ceruloplasmin level, elevated urinary copper, presence of Kayser Fleischer Ring, abnormal hepatic functioning, motor and neuropsychiatric deficits were taken as the key features to identify a WD patient. Neurological features include the

presence of dystonia, tremor, rigidity, gait disturbances, drooling, dysphagia, dysarthria and psychiatric disturbances. Neuropsychiatric signs and symptoms were evaluated by our clinical collaborators using detailed questionnaires and performance based tasks. The mean age  $\pm$  standard deviation (S.D.) of the WD patients selected for our study was  $11.68 \pm 5.61$  years, ranging from 1.5 to 30 years. Age at onset of symptoms of WD was documented based on patients' history and available medical records. The median age  $\pm$  S.D. of the control subjects was  $46 \pm 8.5$  years, ranging from 21 years to 76 years.

***BDNF* and *DRD2* polymorphism:** Genotyping of two polymorphisms in the *BDNF* gene, i.e. rs6265 (p.Val66Met/ c.G196A) and rs56164415 (c.C270T), and rs1800497 (Taq1A; A2/A1 or C/T) of the *DRD2/ANKK1* were performed by polymerase chain reaction (PCR) followed by restriction enzyme digestion. PCR was carried out in 20  $\mu$ L reaction volume using 80 ng of total genomic DNA with Go Taq Green PCR master mix (Promega, India) using specific primers for *BDNF* and *DRD2/ANKK1* (Supplementary Table 1). All the PCR products were electrophoresed in 6% polyacrylamide gel; the amplicons were detected after ethidium bromide (EtBr) staining. The PCR amplicons for *BDNF* (rs6265 and rs56164415) and *DRD2/ANKK1* (rs1800497) were further subjected to restriction digestion using New England Biolab (NEB) enzymes incubating at optimum temperature for 3 hours (Supplementary Table 1). The digested products were electrophoresed on 7% polyacrylamide gel. The genotypes for 25% of the samples were further confirmed using bidirectional Sanger sequencing (ABI3130XL; Applied Biosystems, Foster City, CA). The details of sequencing primers are available on request.

**Statistical Analysis:** Allele and genotype frequencies among patients and controls for the *BDNF* Val66Met and C270T polymorphisms and the *DRD2/ANKK1* Taq1A polymorphism was compared by 2 X 2 contingency Chi-square analysis (95% CI) using JAVASTAT (<http://statpages.info/ctab2x2.html>) and Quantpsy software (<http://www.quantpsy.org/chisq/chisq.htm>). The p value  $<0.05$  from Fisher Exact test was considered to be significant variation within the groups categorized on variable phenotypes of WD patients. A two tailed student's t-test has been performed to compare between the mean ages of the WD patients carrying at least one A (methionine) allele and GG (Val/Val) homozygous genotype for rs6265 (val66met) polymorphism of *BDNF* gene. In addition, logistic regression analysis was performed using Logistic regression calculating page ([statpages.info/logistic.htm](http://statpages.info/logistic.htm)) to evaluate the possible association between independent variables (*BDNF* and *DRD2* genotypes, age and sex) and age at onset of symptoms among WD patients and rigidity. Bonferroni corrections were applied to adjust for multiple testing.

## Results:

Comparing the allelic and genotypic distribution of *BDNF* and *DRD2* polymorphisms among 164 WD patients and 270 controls revealed that the C allele [p – Value: 0.027; OR (95% CI) 0.666 (0.489 - 0.907)] and CC genotype [p–Value: 0.010; OR (95% CI) 0.533 (0.352 – 0.806)] of *BDNF* C270T polymorphism are likely to have protective role, being significantly overrepresented among controls. Our study also revealed a significant association of *DRD2* Taq1A polymorphism, where A1A1 genotype [p–Value: 0.018; OR (95% CI) 2.307 (1.236 – 4.317)] were significantly overrepresented among WD patients compared to controls thus posing risk. No association was found in case of *BDNF* Val66Met polymorphism (Table 1).

None of the selected SNPs of *BDNF* and *DRD2* showed deviation from Hardy-Weinberg equilibrium (HWE) in the control population.

Our WD patient pool comprised 142 cases from eastern part of India (Kolkata) and 22 individuals from the western part of India (Pune). To rule out the possibility of population bias, we run the analyses including all subjects as well as without the smaller western Indian sub-sample, with essentially the same results, with one exception: the Bonferroni-corrected association between the minor *BDNF* C270T allele and WD was no longer significant in the pure eastern Indian sample (Table 1). To further rule out the possibility of a skewed genotype distribution, which may have influenced the overall association with respect to WD, random Bootstrap sampling was carried out within the total WD patient dataset for replicates using 82 samples each. At least eight of the ten replicates presented consistent associations for *BDNF* C270T and *DRD2* Taq1A polymorphisms.

The WD patients included in our study had variable ages of onset ranging from 1.5 to 30 years. The patients also manifested various degrees and severity of hepatic and neurological features, such as serum ceruloplasmin level, urinary copper level, tremor, rigidity, dysarthria, cognitive impairment, and neuropsychiatric disturbances. Aiming to explain this heterogeneity, the *BDNF* and *DRD2* allele and genotype distributions were analyzed within WD patients.

Our analysis revealed that the *BDNF* G allele encoding Valine [p-Value: 0.003; OR (95% CI) 0.323 (0.517 – 0.657)] and GG (Val/Val) genotype [p – Value: 0.012; OR (95% CI) 0.315 (0.134 – 0.734)] were significantly preponderant among WD patients with an age of onset  $\geq 10$  years (Table 2). The WD patients carrying at least one *BDNF* A (Methionine) allele had an earlier age of onset ( $10.16 \pm 4.72$  years) compared to GG (Val/Val) genotype carriers ( $12.45 \pm 5.4$  years) ( $p = 0.015$ ). In addition, the *DRD2* Taq1A the A1 allele [p – Value: 0.003; OR (95% CI) 2.226 (1.324 – 3.750)] and A1A1 genotype [p – Value: 0.018; OR (95% CI) 3.500 (1.326 – 9.482)] were significantly more frequent among WD patients with rigidity compared to those without rigidity (Table 3). However, we did not find any significant difference in the age of onset between these two groups ( $11.84 \pm 5.08$  years vs.  $10.94 \pm 4.65$  years;  $p = 0.317$ ). To investigate whether the *DRD2* Taq1A polymorphism is regulating the rigidity as an independent phenotype we performed the analysis between WD patients with rigidity and WD patients without rigidity along with controls who did not have any personal or family history of neurological diseases. Interestingly the association also held true in this case (Supplementary Table 2).

It is worth mentioning that since we did not have complete records for all the parameters for all the 164 patients, studies of the mentioned endophenotypes were carried out on a subset of WD patients based on the available clinical data (Supplementary Table 3).

Except for the significant associations between *BDNF* Val66Met and age of onset and *DRD2* Taq1A and rigidity, no significant differences in allele or genotype frequencies were found with respect to any of the other clinical phenotypes tested. We did not observe any significant association for the selected variants comparing the WD cases and control with respect to gender (Supplementary Table 4). We did not find any significant association of any allele or genotype of *BDNF* C270T polymorphism with any clinical endophenotype studied amongst WD patients (data not shown).

Logistic regression analysis was performed within the patient sample with different genetic models (additive, dominant and recessive) while analyzing the association between the *BDNF* Val66Met and *DRD2* Taq1A polymorphisms and age at onset of symptoms and rigidity respectively. The analysis revealed *BDNF* Val66Met polymorphism [Additive model p – value: 0.002, OR (95% CI) 0.3530 (0.1817 - 0.6858), Recessive model [p – value: 0.003, OR (95% CI) 0.3153 (0.1448 - 0.6864)] to be significantly associated with age of onset of the disease, while the *DRD2* Taq1A polymorphism [Additive model p – value: 0.029, OR (95% CI) 1.6245 (1.0508 - 2.5115), recessive model p – value: 0.0307, OR (95% CI) 2.4261 (1.0668 - 5.5178)] was significantly associated with rigidity among WD patients. The results of logistic regression analysis have been provided in the Tables 4 and 5.

## Discussion:

Polymorphic variants in the modifier genes are known to influence the phenotype of a disease. Even for monogenic diseases like, cystic fibrosis, Rett syndrome and Wilson's disease, a complex interaction between modifier genes has been reported to alter the expression, severity, and progression of the disease [Grillo et al, 2013, Gallati, 2014]. The clinical heterogeneity of WD has not been explained fully at its molecular level. Movement disorder phenotypes (tremor, rigidity, dystonia, and Parkinsonism) and neuropsychiatric disturbances (cognitive impairments, psychosis, aggressive behaviour, etc.) in WD have been reported to occur due to basal ganglia dysfunctions and/ or neurodegeneration in other related brain structures (Das and Ray, 2006). Previous investigators have highlighted the involvement of *BDNF* in neurodegenerative and neuropsychiatric diseases (Mizui et al, 2014), implicating its potential involvement in WD. In the present study, we found that the C allele and CC genotype of *BDNF* C270T polymorphism play a protective function being significantly overrepresented among control individuals compared to WD patients. It is unlikely to get an overall association of *BDNF* C270T polymorphism with a monogenic disease. However, C270T polymorphism did not reveal any association with any endophenotype studied. Even, the mean age does not vary significantly between the groups bearing different genotype of this polymorphism. This result is similar to our previous finding, where *APOE* variants showed an overall association with the disease (Roy et al, 2017). Besides, other studies have also reported the association of *DMT1* variant with the susceptibility of the disease (Przybyłkowski et al, 2014). In this context, it is likely that C allele or CC genotype of C270T polymorphism may act as a protective factor towards developing hepatic and neurological symptoms among WD patients. Increasing evidence suggests *BDNF* controls glucose metabolism in liver regulating gluconeogenic enzymes (Genzer et al, 2017). This evidence further supports the involvement of *BDNF* function outside the brain. A previous report from *in-silico* analysis revealed that the T allele of C270T polymorphism of *BDNF* could alter binding of transcription factors, i.e. histone H4 transcription factor (HINFP) and Zic family member 3 (ZIC3) leading to deprived expression in the cortical region of brain (Catharius et al, 2005). However, we cannot rule out the possibility that the observed association between *BDNF* C270T and WD is due to mutation in another gene located in the same chromosome as *BDNF* or has emerged by chance.

While studying the distribution of Val66Met polymorphism of *BDNF*, we found that G allele encoding Valine and GG (Val/Val) genotype were significantly more frequent among WD patients with an age of onset beyond 10 years. This possibly suggests that carriers of the G (Valine) allele and GG (Val/Val) genotype are less prone to develop WD symptoms at an earlier age. The Val66Met polymorphism is present in the pro-domain region of *BDNF*.



BDNF pro-peptide acts through p75 neurotrophin receptor facilitating long-term depression inducing apoptosis (Mizui et al, 2017). This may underlie the pathogenesis of neurodegeneration in WD. Previously, it has been reported that the Val66Met polymorphism impairs intracellular trafficking losing its interaction with the sortilin receptor complex (Mizui et al, 2014). In vitro cell based studies revealed that substitution of the methionine residue at this position leads to decreased distribution to the dendrites and impaired secretion (Egan et al, 2003). Functional magnetic resonance imaging (f-MRI) studies have shown that Val/Val carriers have a significantly higher level of hippocampal activation compared to Val/Met carriers. The *BDNF* Met allele affects glucose metabolism in brain regions like the hippocampus and the temporal parietal and occipital cortex (Xu et al, 2010). In mammals, BDNF is highly expressed in hippocampus, hence, neurodegeneration and cognitive dysfunctions observed among WD patients may be attributed to methionine substitution at this position (Mizui et al, 2017). The molecular mechanism underlying the earlier onset of WD symptoms remains elusive. Proper BDNF signalling in hepatocytes may promote fatty acid oxidation and glycogen storage (Genzer et al, 2017). Considering these evidences it is suggested that the presence of wild type G (Val) allele or GG (Val/Val) genotype may delay the onset of symptoms among WD patients.

In this study the minor allele A1 and A1A1 genotype of Taq1A polymorphism of *DRD2* gene were significantly preponderant among WD patients compared to healthy controls. Further, analyzing the distribution of allele and genotype for Taq1A polymorphism of *DRD2* within WD patients, we found that A1 allele and A1A1 genotypes act as a risk factor for developing rigidity. The present study is in accordance with previous reports suggesting that A1 allele and/or A1A1 genotype may serve as a risk factor for developing neurodegenerative disorders like Parkinson's and Alzheimer's disease (Noble et al, 2003). Similar positive associations between the Taq1A polymorphism and alcohol dependence, addiction, schizophrenia and attention deficit hyperactivity disorder (ADHD) have been found (Noble, 2000, Glatt et al, 2003, Rowe et al, 1999). In-vivo studies have also revealed association of the A1 allele with lower glucose metabolism in the dopaminergic neurons of the human brain (Rowe et al, 1999). The A1 allele of this polymorphism has been associated with lower dopamine D2 receptor expression in the striatum of post mortem brain (Noble et al, 2003). Most of the movement disorders and neuropsychiatric presentations of WD possibly occur due to defects in basal ganglia and prefrontal cortex. Radiological evidence and studies with post mortem brain revealed low dopamine D2receptor expression in the striatum of WD patients (Oder et al, 1996). This leads us to hypothesize that decreased *DRD2* expression in the striatum of the carriers of the *DRD2* Taq1 A1 allele and A1A1 genotype among WD patients underlie the cause of poor dopaminergic transmission resulting in rigidity symptoms. The mean ages of WD patients with and without rigidity did not vary significantly, which excludes the possibility of a prominent age factor to be involved in the development of rigidity among patients age 30 or below.

Unavailability of clinical data for some of the patients restricted our analyses on smaller subsets of WD patients, which is a limitation of the study. Besides, we could not perform any further analysis based on association with the rarer clinical presentations, such as schizophrenia, depression and impulsiveness among these WD patients due to lack of follow-up.

The patient pool included in this study contains heterogeneous mutation profiles including compound heterozygous conditions. Although the c.813C>A, Cys271X mutation in the

*ATP7B* gene is common among our patient cohort, the number of patients was too low to compare the effect of *BDNF* and *DRD2* polymorphisms solely on the mutational background. Apart from prognostic benefits of studying modifier genes, it also leads us to the understanding of the etiology of the disease, where a single gene alone may not play a major role. Rather, the varying clinical manifestations are likely the result of complex interactions between different genes connected to biochemical pathways involved with the pathogenesis of the disease triggered by a single gene (*ATP7B*) in a monogenic disease.

### **Conclusion:**

In conclusion our findings suggest that the *BDNF* C270T C allele may be associated with the occurrence of WD, the *BDNF* Val66Met Val allele and its homozygous genotype to serve as a protective factor delaying the age of onset. On the other hand in case of *DRD2* Taq1A, A1 allele and its homozygous genotype may be a risk factor for developing rigidity among patients with WD. However, our hypotheses need to be independently replicated.

**Acknowledgment:** We are thankful to the Wilson's disease patients and their family members for participating in this study. The study was partially supported by the funding from Department of Science & Technology Cognitive Sciences Research Initiative (DST-CSRI), Govt. of India. SR is supported by UGC-JRF fellowship from UGC, Govt. of India, PP is supported by University Research Fellowship and SG had been supported by a fellowship from DST-SERB, Govt. of India.

### **Conflict of Interest:**

Authors declare no conflict of interest with respect to this article.

### **Documentation of the authors' contribution:**

SR: PCR-RFLP and sequencing of *BDNF* (Val66Met and C270T) and *DRD2* gene among WD patients (Val66Met, 164 samples, C270T 104 samples, *DRD2*, 142 samples) analysis of the result and drafting of the manuscript.

PP: PCR-RFLP of *BDNF* Val66Met and C270T among 270 controls

SG: PCR-RFLP for *BDNF*, C270T and *DRD2* among WD samples (*BDNF*, 60 samples, *DRD2*, 20 samples)

SB: PCR-RFLP for *DRD2* among control samples (50 samples).

MSG: Involved in study design, experimental details and writing of the manuscript.

KR: A senior investigator in WD project provided intellectual input and preparation of the manuscript.

JR: Principle Investigator of the project provided intellectual input and involved in preparation of the manuscript.

SKD: Patient recruitment, clinical evaluation and blood sample collection from patients from Kolkata area

PKG: Patient recruitment, clinical evaluation and blood sample collection from patients from Kolkata area

AB: Patient recruitment, clinical evaluation and blood sample collection from patients from Pune area

## References:

- Das, SK., Ray K. (2006). Wilson's disease: an update. *Nature Review Neurology*, 2(9):482.
- Todorov, T., Balakrishnan, P., Savov, A., Socha, P., Schmidt, HH. (2016). Intragenic Deletions in ATP7B as an Unusual Molecular Genetics Mechanism of Wilson's Disease Pathogenesis. *PloS one*, 11(12), e0168372.
- Kegley, KM., Sellers, MA., Ferber, MJ., Johnson, MW., Joelson, DW., Shrestha, R. (2010). Fulminant Wilson's disease requiring liver transplantation in one monozygotic twin despite identical genetic mutation. *American Journal of Transplantation*, 10(5), 1325-9.
- Kieffer, DA., Medici, V., (2017) Wilson disease: At the crossroads between genetics and epigenetics—A review of the evidence. *Liver Research*. 1(2), 121-130.
- Mizui, T., Tanima, Y., Komatsu, H., Kumanogoh, H., Kojima, M., (2014). The biological actions and mechanisms of brain-derived neurotrophic factor in healthy and disordered brains. *Neuroscience and Medicine*, 5(04), 183.
- Mizui, T., Ishikawa, Y., Kumanogoh, H. et al, (2015). BDNF pro-peptide actions facilitate hippocampal LTD and are altered by the common BDNF polymorphism Val66Met. *Proceedings of the National Academy of Sciences*, 112(23), E3067-E3074.
- Travaglia, A., La Mendola, D., Magrì, A., Nicoletti, VG., Pietropaolo, A., Rizzarelli, E. (2012). Copper, BDNF and Its N-terminal Domain: Inorganic Features and Biological Perspectives. *Chemistry- A European Journal*, 18(49), 15618-31.
- Autry, AE., Monteggia, LM. (2012). Brain-derived neurotrophic factor and neuropsychiatric disorders. *Pharmacological Reviews*, 64(2), 238-58.
- Mizui, T., Ohira, K., Kojima, M., (2017). BDNF pro-peptide: a novel synaptic modulator generated as an N-terminal fragment from the BDNF precursor by proteolytic processing. *Neural Regeneration Research*, 12(7), 1024.
- Watanabe, Y., Nunokawa, A., Someya, T. (2013). Association of the BDNF C270T polymorphism with schizophrenia: A meta-analysis. *Psychiatry and Clinical Neuroscience*, 67(2), 123-5.
- Cartharius, K., Frech, K., Grote, K., et al. (2005). MatInspector and beyond: promoter analysis based on transcription factor binding sites. *Bioinformatics*, 21(13), 2933-42.
- Klein, C., Brin, M.F., Kramer, P., et al (1999) Association of a missense change in the D2 dopamine receptor with myoclonus dystonia. *Proceedings of the National Academy of Science. USA*, 96, 5173–76
- Nyberg, P., Gottfries, CG., Holmgren, G., Persson, S., Roos, BE., Winblad, B. (1982). Advanced catecholaminergic disturbances in the brain in a case of Wilson's disease. *Acta Neurologica Scandinavica*, 65(1), 71-5

- De Vries,DJ., Sewell, RB., Beart, PM. (1986). Effects of copper on dopaminergic function in the rat corpus striatum. *Experimental Neurology*, 91(3), 546-58.
- Oder, W., Brücke, T., Kollegger, H., Spatt, J., Asenbaum, S., Deecke, L. (1996). Dopamine D2 receptor binding is reduced in Wilson's disease: Correlation of neurological deficits with striatal 123 I-Iodobenzamide binding. *Journal of Neural Transmission*, 103(8-9), 1093-103
- Westermarck, K., Tedroff, J., Thuomas, KA. , et al. (1995). Neurological Wilson's disease studied with magnetic resonance imaging and with positron emission tomography using dopaminergic markers. *Movement disorders*, 10(5), 596-603.
- Członkowska, A., Jachowicz-Jeszka, J., Członkowski, A. (1987). [3H] Spiperone binding to lymphocyte in extrapyramidal disease and in aging. *Brain, Behavior and Immunity*, 1(3), 197-203.
- Noble, EP.(2003). D2 dopamine receptor gene in psychiatric and neurologic disorders and its phenotypes.American Journal of Medical Genetics Part B: Neuropsychiatric Genetics,116(1), 103-25.
- Blum, K., Badgaiyan, RD., Dunston, GM., et al. (2017). The DRD2 Taq1A A1 Allele May Magnify the Risk of Alzheimer's in Aging African-Americans. *Molecular Neurobiology*,1-11.
- Gupta, A., Chattopadhyay, I., Mukherjee, S., Sengupta, M., Das, SK., Ray, K. (2010).A novel COMMD1 mutation Thr174Met associated with elevated urinary copper and signs of enhanced apoptotic cell death in a Wilson Disease patient. *Behavioral and Brain Functions*. 2010. 6(1):33.
- Roy, S., Ganguly, K., Pal,P., et al. (2017). Influence of Apolipoprotein E polymorphism on susceptibility of Wilson disease. *Annals of Human Genetics*, 82(2), 53-59.
- Mukherjee, S., Dutta, S., Majumdar,S.,et al. (2014). Genetic defects in Indian Wilson disease patients and genotype–phenotype correlation. *Parkinsonism and Related Disorder*, 20(1), 75-81.
- Sternlieb, I. (1990). Perspectives on Wilson's disease. *Hepatology*, 12(5), 1234-1239.
- Grillo, E., Rizzo, CL., Bianciardi, L.et al. (2013).Revealing the complexity of a monogenic disease: Rett syndrome exome sequencing.PLoSOne.8(2), e56599.
- Gallati S (2014). Disease-modifying genes and monogenic disorders: experience in cystic fibrosis. *The Application of Clinical Genetics*. 7:133–46.
- Przybyłkowski, A., Gromadzka, G., Członkowska, A. (2014).Polymorphisms of metal transporter genes DMT1 and ATP7A in Wilson's disease. *Journal of Trace Elements in Medicine and Biology*,28(1), 8-12.
- Genzer, Y., Chapnik, N., Froy, O. (2014).Effect of brain-derived neurotrophic factor (BDNF) on hepatocyte metabolism. *International Journal of Biochemistry and Cell Biology*, 88, 69-74.
- Egan,MF., Kojima, M., Callicott,JH,et al. (2003) The BDNF val66met polymorphism affects activity-dependent secretion of BDNF and human memory and hippocampal function. *Cell*, 112(2), 257-69.

Xu C, Wang Z, Fan M,et al. (2010). Effects of BDNF Val66Met polymorphism on brain metabolism in Alzheimer's disease. *NeuroReport*. 21(12), 802.

Noble, EP. (2000). Addiction and its reward process through polymorphisms of the D2 dopamine receptor gene: a review. *European Psychiatry*,15(2), 79-89.

Glatt,SJ.,Faraone, SV., Tsuang, MT. (2003). Meta-analysis identifies an association between the dopamine D2 receptor gene and schizophrenia. *Molecular Psychiatry*. 8(11), 911.

Rowe,DC., Van den Oord, EJ., Stever,C.,et al. (1999).The DRD2 TaqI polymorphism and symptoms of attention deficit hyperactivity disorder. *Molecular Psychiatry*.4(6), 580.

Munafo, MR., Matheson,IJ., Flint, J. (2007). Association of the DRD2 gene Taq1A polymorphism and alcoholism: a meta-analysis of case–control studies and evidence of publication bias. *Molecular Psychiatry*, 12(5), 454.

Table 1: Frequency of allelic and genotypic distribution of *BDNF* and *DRD2* polymorphisms among WD patients and controls

Gene/ Polymorphism	Allele/ Genotype	WD Patients n = 164 (%)	Control n = 270 (%)	OR (95% CI)	p - Value
<i>BDNF</i> (Val66Met)	G	254 (77.43)	407 (75.37)	1.122 (0.801 – 1.573)	0.512 <sup>a</sup>
	A	74 (22.56)	133 (24.63)		
	GG	101 (61.58)	152 (56.29)	1.124 (0.822 – 1.886)	0.316
	GA	52 (31.70)	103 (38.15)	0.753 (0.489 – 1.158)	0.181
	AA	11 (6.70)	15 (5.56)	1.222 (0.509 – 2.907)	0.678
<i>BDNF</i> (C270T)	C	215 (65.54)	400 (74.07)	0.666 (0.489 – 0.907)	0.027 <sup>b*</sup>
	T	113 (34.45)	140 (25.92)		
	CC	65 (39.63)	149 (55.18)	0.533 (0.352 – 0.806)	0.006 *
	CT	85 (51.83)	102 (37.78)	1.772 (1.174 – 2.677)	0.025 *
	TT	14 (8.54)	19 (7.04)	1.233 (0.566 – 2.669)	0.579
<i>DRD2</i> (Taq1A)	A2	201 (61.28)	379 (70.18)	1.376 (1.018 – 1.859)	0.108 *
	A1	127 (38.71)	161 (29.81)		
	A2A2	72(42.07)	132 (48.88)	0.818 (0.543 – 1.231)	0.323
	A2A1	63 (38.14)	115 (42.59)	0.841 (0.554 – 1.274)	0.421
	A1A1	29 (19.51)	23 (8.51)	2.307 (1.236 – 4.317)	0.018 *
Gene/ Polymorphism	Allele/ Genotype	Eastern Indian WD Patients n = 142 (%)	Control n = 270 (%)	OR (95% CI)	p - Value
<i>BDNF</i> (Val66Met)	G	220 (77.46)	407 (75.37)	0.890 (0.624 – 1.268)	0.548 <sup>a</sup>
	A	64 (22.53)	133 (24.63)		
	GG	88 (61.97)	152 (56.29)	1.265 (0.818 – 1.959)	0.294
	GA	44 (30.98)	103 (38.15)	0.728 (0.461 – 1.147)	0.161
	AA	10 (7.04)	15 (5.56)	1.288 (0.521 – 3.143)	0.525
<i>BDNF</i> (C270T)	C	188 (66.19)	400 (74.07)	0.685 (0.496 – 0.948)	0.057 <sup>b*</sup>
	T	96 (33.81)	140 (25.92)		
	CC	58 (40.84)	149 (55.18)	0.561 (0.364 – 0.864)	0.021*
	CT	72 (50.70)	102 (37.78)	1.694 (1.100 – 2.610)	0.036 *
	TT	12 (8.45)	19 (7.04)	1.219 (0.538 – 2.738)	0.695
<i>DRD2</i> (Taq1A)	A2	172 (60.56)	379 (70.18)	1.533 (1.121 – 2.095)	0.018 <sup>c*</sup>
	A1	112 (39.43)	161 (29.81)		
	A2A2	57(40.14)	132 (48.88)	0.701 (0.455 – 1.080)	0.097
	A2A1	58 (40.84)	115 (42.59)	0.931 (0.603 – 1.435)	0.754
	A1A1	27 (19.01)	23 (8.51)	2.521 (1.331 – 4.784)	0.009 *

WD, Wilson's disease; *BDNF*, brain derived neurotrophic factor; *DRD2*, dopamine receptor *D2*; Val66Met, Valine66Methione; OR, Odds Ratio; 95% CI, 95% Confidence Interval.

p – Value < 0.05 considered statistically significant

a, A vs G allele among WD patients and Controls for *BDNF* Val66Met

b, Cvs T allele among WD patients and Controls for *BDNF* C270T

c, A1 vs A2 allele among WD patients and Controls for *DRD2*TaqIA

\*Bonferroni adjusted *p* – values for multiple testing



Table 2: Frequency of Allelic and genotypic distribution of *BDNF* Val66Met polymorphism among WD patients with early ( $\leq 10$  years) and late onset ( $> 10$  years) of symptoms

Gene	Allele/ Genotype	Age of Onset		OR (95% CI)	p - Value
		Early Onset ( $\leq 10$ years) n = 61	Late Onset ( $> 10$ years) n = 63		
<i>BDNF</i> Val66Met	G	86 (70.49)	111 (88.09)	0.323 (0.517 – 0.657)	0.003 **
	A	36 (29.50)	15 (11.90)		
	GG	32 (46.37)	49 (77.77)	0.315 (0.134 – 0.734)	0.012 **
	GA	22 (31.88)	13 (20.63)	2.170 (0.905 – 5.247)	0.073
	AA	7 (10.14)	1 (1.58)	8.037 (0.941 – 179.42)	0.093 **

*BDNF*, brain derived neurotrophic factor; OR, Odds Ratio; CI, Confidence Interval, p- Value < 0.05 considered statistically significant.

\*\*Bonferroni adjusted p – values for multiple testing

Table 3: Frequency of Allelic and genotypic distribution of *DRD2/ANKK1*Taq1A polymorphism among WD patients with and without rigidity

Gene/ Polymorphism	Allele/ Genotype	Rigidity		OR (95% CI)	p - Value
		Present n = 69 (%)	Absent n = 72 (%)		
<i>DRD2</i> /Taq1A	A2	66 (47.83)	42 (29.16)	2.226 (1.324 – 3.750)	0.003 ***
	A1	72 (52.17)	102 (70.83)		
	A2A2	24 (34.78)	38 (52.77)	0.477 (0.228 – 0.992)	0.126 ***
	A2A1	24 (34.78)	26 (36.11)	0.994 (0.446 – 1.994)	1.000
	A1A1	21 (30.43)	8 (11.11)	3.500 (1.326 – 9.482)	0.018 ***

WD, Wilson's disease; OR, Odds Ratio; 95% CI, Confidence Interval; *DRD2*, dopamine receptor D2

p – Value < 0.05 considered statistically significant

\*\*\*Bonferroni adjusted p – values for multiple testing

Independent Variables	Model	Genotype	p – Value	OR (95% CI)
<i>BDNF</i> Val66Met	Additive	GG vs GA vs AA	0.002	0.3530 (0.1817 - 0.6858)
	Dominant	GG + GA vs AA	0.054	0.1244 (0.0148 - 1.0437)
	Recessive	GG vs GA + AA	0.003	0.3153 (0.1448 - 0.6864)

Table 4: Logistic regression analysis of independent variable to age of onset for WD patients

*BDNF*, brain derived neurotrophic factor; OR, Odds Ratio; CI, Confidence Interval

*p* – Value < 0.05 considered statistically significant.

Independent Variables	Model	Genotype	p – Value	OR (95% CI)
<i>DRD2</i> <i>Taq1A</i>	Additive	GG vs GA vs AA	0.029	1.6245 (1.0508 - 2.5115)
	Dominant	GA + AA vs GG	0.094	1.7736 (0.9008 - 3.4922)
	Recessive	AA vs GA + GG	0.0307	2.4261 (1.0668 - 5.5178)

Table 5: Logistic regression analysis of independent variable to rigidity for WD patients

*DRD2*, Dopamine Receptor *D2*; OR, Odds Ratio; CI, Confidence Interval

*p* – Value < 0.05 considered statistically significant.

Gene	Polymorphisms	Amplicon length	Primer Sequences	PCR conditions	Restriction Enzymes	Digestion patterns
<i>BDNF</i>	rs 6265/ G196A/ Val66Met	615 bp	Forward Primer : 5' AAACATCCGAGGACAAGGT3' Reverse Primer : 5'GCCAGCCAATTCTCTTTTG3'	56 °C (30'-30'-30') X 35 cycles	Pml I	G = 490 + 125 bp A = 615 bp
	rs 56164415/ C270T	313 bp	Forward Primer : 5' GGGGGCTTTAATGAGACACC 3' Reverse Primer : 5' GGAGAAAAC TCCCAAGAG3'	54 °C (30'-30'-30') X 35 cycles	Hpy188III	C = 313 bp T = 189 + 124 bp
<i>DRD2</i>	rs1800497/ Taq1A	338 bp	Forward Primer: 5'GAGGAGCACCTTCCTGAGTG 3' Reverse Primer: 5' CTGGGATTACAGACGTGAG3'	66° - 56° C touchdown; 35 cycles	Taq I	G = 132 + 206 bp A = 338 bp

Supplementary Table 1: PCR and RFLP conditions for selected polymorphisms

Supplementary Table 2: Frequency of Allelic and genotypic distribution of *DRD2/ANKK1* Taq1A polymorphism among WD patients with rigidity and WD patients without rigidity along with the healthy controls.

Gene/ Polymorphism	Allele/ Genotype	Rigidity		OR (95% CI)	p - Value
		Present n = 69 (%)	Absent n = 342 (%)		
<i>DRD2</i> /Taq1A	A2	66 (47.83)	421 (61.54)	1.746 (1.189 – 2.565)	0.009*
	A1	72 (52.17)	263 (38.45)		
	A2A2	24 (34.78)	170 (49.70)	0.540 (0.304 – 0.955)	0.075*
	A2A1	24 (34.78)	141 (41.22)	0.760 (0.427 – 1.347)	0.348
	A1A1	21 (30.43)	31 (9.06)	4.389 (2.225 – 8.645)	0.000003*

WD, Wilson's disease; OR, Odds Ratio; CI, Confidence Interval; *DRD2*, dopamine receptor D2

\*Bonferroni adjusted *p*- values for multiple testing.

Supplementary table 4: Allele and genotype distribution for *BDNF* and *DRD2* polymorphisms among the WD patients and Control with respect to Gender.

Gene/ Polymorphism	Allele/ Genotype	WD Patients Male n = 104	Control Male n = 212	OR (95% CI)	p - value
<i>BDNF</i> Val66Met	G	165 (79.32)	322 (75.94)	0.823 (0.539 – 1.254)	0.366
	A	43 (20.67)	102 (24.05)		
	GG	70 (67.30)	124 (58.49)	1.490 (0.885 - 2.513)	0.140
	GA	25 (24.03)	75 (35.37)	0.566 (0.322 – 0.993)	0.200 <sup>†</sup>
	AA	9 (8.65)	13 (6.132)	1.450 (0.549 – 3.783)	0.481
<i>BDNF</i> C270T	C	134 (64.42)	314 (74.05)	0.745 (0.506 – 1.097)	0.125
	T	74 (35.57)	110 (25.94)		
	CC	41(39.42)	117 (55.18)	0.528 (0.319 – 0.875)	0.060 <sup>†</sup>
	CT	52 (50.0)	80 (37.73)	1.650 (0.999 – 2.727)	0.200 <sup>†</sup>
	TT	11 (10.57)	15 (7.07)	1.553 (0.637 – 3.756)	0.285
<i>DRD2</i> Taq1A	A2	128 (61.53)	292 (68.86)	1.383 (0.963 – 1.985)	0.073
	A1	80 (38.46)	132 (31.13)		
	A2A2	45 (43.27)	101 (47.64)	0.838 (0.308 – 1.381)	0.474
	A2A1	38 (36.53)	90 (42.45)	0.780 (0.467 – 1.301)	0.332
	A1A1	21 (20.19)	21 (9.90)	2.301 (1.135 – 4.667)	0.070 <sup>†</sup>
Gene/ Polymorphism	Allele/ Genotype	WD Patients Female n = 60	Control Female n = 58	OR (95% CI)	p - value
<i>BDNF</i> Val66Met	G	89 (74.16)	85 (73.27)	0.955 (0.514 – 1.776)	0.884
	A	31 (25.83)	31 (26.72)		
	GG	31 (51.67)	29 (50.0)	1.069 (0.487 – 2.347)	1.000
	GA	27 (45.0)	27 (46.55)	0.939 (0.427 – 2.067)	1.000
	AA	2 (3.33)	2 (3.45)	0.966 (0.093 - 10.018)	1.000
<i>BDNF</i> C270T	C	81 (67.50)	86 (74.13)	1.380 (0.756 – 2.525)	0.317
	T	39 (32.50)	30 (25.86)		
	CC	24 (40.0)	32 (55.17)	0.542 (0.244 – 1.199)	0.140
	CT	33 (55.0)	22 (37.93)	2.000 (0.900 – 4.467)	0.068
	TT	3 (5.0)	4 (6.89)	0.711 (0.119 – 3.997)	0.715
<i>DRD2</i> Taq1A	A2	79 (65.83)	87 (75.0)	1.557 (0.852 – 2.849)	0.154
	A1	41 (34.16)	29 (25.0)		
	A2A2	27 (45.0)	31 (53.45)	0.713 (0.323 – 1.567)	0.461
	A2A1	25 (41.66)	25 (43.10)	0.943 (0.425 – 2.089)	1.000
	A1A1	8 (13.33)	2 (3.44)	4.308 (0.790 – 30.908)	0.095

WD, Wilson's disease; OR, Odds Ratio; 95% CI, Confidence Interval; *BDNF*, brain derived neurotrophic factor; *DRD2*, dopamine receptor D2

p – Value < 0.05 considered statistically significant

<sup>†</sup>Bonferroni adjusted p – value for multiple testing